

LIPASE-CATALYZED SYNTHESIS OF OLEIC ACID ESTERS OF POLYETHYLENE GLYCOL 400

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Summary

Quantitative esterification of polyethylene glycol (PEG) 400 using oleic acid and Lipozyme was achieved in hexane. The effects of temperature, nature of acyl donor, substrate ratio, enzyme quantity and reaction time upon PEG esterification were examined. Nearly selective production of either PEG monooleate or PEG dioleate was achieved. Lipozyme was still 80% active after five reaction cycles.

Introduction

Cosmetic, nutritional, and pharmaceutical products often contain mono- and diesters of diols (Als and Krog, 1990). Polyethylene glycol (PEG) esters of fatty acids are low-foaming, non-ionic surfactants which are used in diverse applications such as de-inking agents for used newsprint, emulsifiers in pesticides, medicinal diluents, and cleanser formulations (Reck, 1989). In this paper we describe the results of our investigation of the enzymatic synthesis of the oleic acid mono- and diesters of PEG 400 which are utilized extensively as lubricants in the metalworking industry (Rakoff and Nidock, 1989). Contrary to the chemical synthesis of these compounds, where high temperatures and toxic catalysts are often involved, enzymatic conversions appear to be the more attractive and energy-efficient alternative. In addition to the moderate conditions under which enzymes are active, the product purity resulting from substrate selectivity and the absence of by-product formation are other advantages that arise when utilizing enzymes as catalysts.

Lipases are known and widely used not only for their capacity to hydrolyze esters in aqueous media but also for their ability to form ester bonds in aqueous and organic solvents (Zaks and Klivanov, 1984; Wong, 1989; Klivanov, 1989). Immobilization of a biocatalyst often leads to increased stability and activity, and facilitates recovery of the catalyst for reuse (Malcata *et al.*, 1990; Powell, 1990; Wehtje *et al.*, 1993). For these reasons we chose Lipozyme®, an immobilized form of *Rhizomucor miehei* lipase as esterification catalyst. The least toxic organic solvents of choice for ester synthesis in the presence of

lipase are non-polar, non-water stripping solvents i.e., hydrocarbons such as hexane, where the highest enzymatic activity and stability have been recorded (Gorman and Dordick, 1992) and where control of the water content is easiest to achieve. However, the relative insolubility of diols, and in particular PEG, in non-polar solvents raises the problem of possible low esterification rates (Eigtved *et al.*, cited by Berger *et al.*, 1992). Nonetheless, the mono and diesters of PEG are readily soluble in these solvents and thus displacement of the reaction equilibrium towards total esterification of PEG is rendered possible. For these reasons we examined the esterification of PEG 400 with oleic acid into PEG 400 monooleate and PEG 400 dioleate using Lipozyme in hexane.

Materials and Methods

Chemicals: Polyethylene glycol 400 (approx. 95%) was purchased from Fluka (Buchs, Switzerland). PEG 400 mono and dioleate standards were kind gifts from Stepan Chemical (Maywood, NJ) and were purified by reverse phase thin-layer chromatography on LKC 18 plates (Whatman, Hillsboro, OR). Oleic acid, oleic acid anhydride, triolein, and methyl oleate were purchased from Sigma (St. Louis, MO) and were all 99% pure. Immobilized lipase from *Rhizomucor miehei* (Lipozyme IM 20) came from Novo (Danbury, CT). All solvents were of HPLC grade and came from Burdick and Jackson (Muskegon, MI).

Esterification of PEG 400 with oleic acid in the presence of Lipozyme and analysis of product formation: Typically, Lipozyme (0.1 g) was placed in a screw-capped test tube to which oleic acid (0.35 g, 1.24 mmoles) in 5 mL hexane and PEG 400 (0.1 g, 0.25 mmoles) had been added. The teflon-sealed, capped tube was shaken in a controlled environment incubator shaker (New Brunswick Scientific, New Brunswick, NJ) set at the desired temperature for 50 hrs, at 300 rpm. Upon completion of the reaction the contents of the tube were filtered over Whatman # 12 paper, and the tube was rinsed with 25 mL methylene chloride. Aliquots of the filtrate were dried under nitrogen, redissolved in 1 mL of methanol, and analyzed by high-performance liquid chromatography.

Thin-layer chromatography (TLC) of PEG 400, PEG 400 monooleate, and PEG 400 dioleate: Samples were applied 2.5 cm from the lower edge of LKC 18 plates (Whatman, Hillsboro, OR) which were then developed to the top in 100% methanol. Dragendorff's reagent (Thoma, 1964), a reagent specific for PEG and PEG derivatives, was then sprayed onto the plates and the resulting orange spots permitted the visualization of PEG and its esters.

High-performance liquid chromatography (HPLC) of PEG 400, PEG 400 monooleate, and PEG 400 dioleate: Baseline separation of the reactants (PEG and oleic acid) and of the products of the reaction (PEG monooleate and PEG dioleate) was accomplished using a reverse-phase C18 Adsorbosphere 5U column (250 mm x 4.6 mm ID, Alltech, Deerfield, IL). Methanol (100%) under constant nitrogen sparging was the mobile phase, and the flow-rate was 0.75 mL/min. Detection and quantification of the compounds were done with an ELSD II A evaporative light-scattering mass detector (Varex, Burtonsville, MD) set at 120 °C. The quantities of reactants and products were determined by comparing the area units obtained for each of these compounds with those of a corresponding calibration curve obtained with pure reference standards.

Results

Acylation was attempted using oleic acid, methyl oleate, triolein, and oleic acid anhydride as acyl donors at 30 °C and 42 °C. The best acylation was achieved at the latter

temperature using oleic acid and oleic anhydride as the acyl donor (Table 1). The acylation attempts at 30 °C gave essentially no esterification of PEG with methyl oleate or triolein, whereas use of oleic acid resulted in only 70% PEG esterification. In parallel to this study, we also looked at the effect of water in the system: water being one of the by-products of the esterification reaction, and a substrate in the competing hydrolysis reaction, the results from assays that were performed in hexane dried overnight with molecular sieves ("dry hexane") were compared to those obtained with water-saturated hexane ("wet hexane"). With either oleic acid or oleic anhydride essentially complete acylation occurred in dry hexane (Table 1). Lower activities were observed with wet hexane in the cases of oleic acid and triolein (Table 1). Oleic acid was chosen as acyl donor for the subsequent experiments.

Table 1. Acylation of PEG 400 by Lipozyme using Various Acyl Donors^a.

Sample	PEG Monooleate	PEG Dioleate	Acylation Yield
	% ^b		
Oleic Acid/Dry Hexane	23.4 ± 0.1	76.6 ± 0.1	100.0 ± 0.2
Oleic Acid/Wet Hexane	27.1 ± 1.0	31.6 ± 1.7	58.7 ± 2.7
Oleic Anhydride/Dry Hexane	22.1 ± 2.3	77.9 ± 1.7	100.0 ± 4.0
Oleic Anhydride/Wet Hexane	15.8 ± 0.4	84.2 ± 0.4	100.0 ± 0.8
Methyl Oleate/Dry Hexane	3.8 ± 0.3	9.4 ± 0.2	13.2 ± 0.5
Methyl Oleate/Wet Hexane	6.3 ± 3.0	7.8 ± 3.3	14.1 ± 6.3
Triolein/Dry Hexane	23.3 ± 0.2	46.2 ± 3.3	69.5 ± 3.5
Triolein/Wet Hexane	8.7 ± 4.6	23.1 ± 4.7	31.8 ± 9.3

^a The concentrations of oleic acid, oleic anhydride, methyl oleate, and triolein were 150, 100, 150, and 50 mM, respectively. The concentration of PEG 400 was 50 mM. The amount of Lipozyme used was 0.1 g. All experiments were performed for 150 hrs in 5 mL of solvent. The temperature was 42 °C, and the reaction vessels were shaken at 300 rpm.

^b All experiments were conducted twice in duplicate. The listed values are the averages and have been normalized with respect to the results obtained with oleic acid in dry hexane (100%).

To examine the effect of the removal of water produced during the reaction, upon the reaction profile, time course studies were conducted at 42 °C in the presence or absence of molecular sieves. Similar levels of PEG esterification were obtained. In the presence of molecular sieves, a significant amount (60%) of PEG (50 mM) was esterified by oleic acid (150 mM) after 75 hrs of reaction. The product was mostly PEG dioleate (Fig. 1). In the absence of molecular sieves, a similar conversion of PEG was achieved after 50 hrs (results not shown) in fresh hexane. Thus, it appears that under these conditions the removal of water by means of molecular sieves does not increase the rate or extent of acylation. The

use of molecular sieves was therefore abandoned and all following experiments were performed in fresh hexane. The effect of the molar ratio, R , of [oleic acid]/[PEG] on ester production at 42 °C was also examined (Fig. 2): Results in the literature (Berger *et al.*, 1992) indicate that by varying the molar ratio of acyl donor to acyl acceptor, it is possible to modulate the extent of diol esterification into exclusively monoester (low R value) or diester (high R value) formation. Maximal PEG transformation to PEG dioleate, resulting in an approx 85% yield of diester, occurred at R equals to 5. In their attempts to esterify short chain glycols, Okumura *et al.* (1979) used much higher concentrations of glycol in an aqueous system containing non-immobilized lipases (0.1 mL enzyme solution, 30 units): among the glycols they tested, trimethylene glycol produced the highest ester yield (roughly 80% conversion was obtained for all lipases tested), but a diol concentration of 70.5%, in a final reaction mixture volume of about 7.3 mL, was required. In our system only 0.1 g of PEG (2% approx.) in a final volume of about 5.2 mL was needed to ensure its efficient transformation by oleic acid.

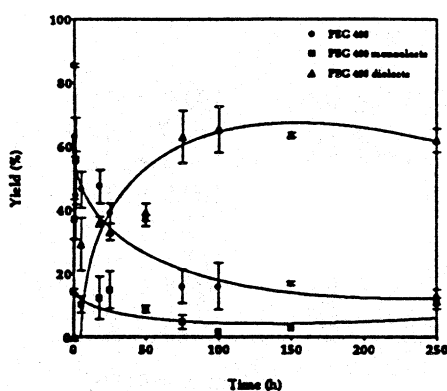


Fig. 1 Time course study of the production of PEG 400 esters at 42 °C in the presence of molecular sieves, 50 mM PEG 400, 150 mM oleic acid.

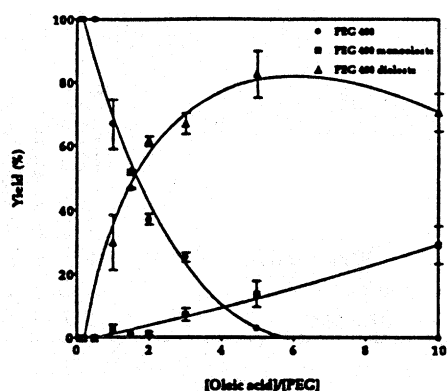


Fig. 2 Effect of substrate ratio on PEG 400 acylation by Lipozyme at 42 °C after 50 hrs incubation.

At the lowest R value assayed here (0.1) no significant accumulation of PEG monooleate was seen. When the R value was lowered even further ($R=0.005$, data not shown), no esterification of PEG was noticed. However, it was found that by using shorter incubation times and $R=5$ at 42 °C, PEG monooleate was mainly produced (approx. 70%) (Fig. 3). In the hope of increasing esterification rates, several experiments similar to those done at 42 °C were conducted at 50 °C as lipase activity in organic solvent is stimulated at higher temperatures (Klibanov and Fitzpatrick, 1990; Malcata *et al.*, 1990). Also better mixing of the substrates, rendered less viscous at higher temperature, might occur leading to higher esterification rates (Dordick, 1991). However, a higher R value ($R=10$) was needed at 50 °C to ensure complete esterification of PEG (results not shown). Furthermore, time

courses performed at 50 °C showed that total acylation of PEG was not achieved until 100 hrs and a loss of selectivity was observed (55% of PEG monooleate was produced at this point) (Fig. 4).

The effect of enzyme concentration at 42 °C upon PEG acylation was also examined, but an experiment in which the amount of Lipozyme ranged from 0.01 g to 1 g showed that neither higher nor lower amounts of Lipozyme gave better reaction rates than that of 0.1 g, nor increases in monooleate production (results not shown).

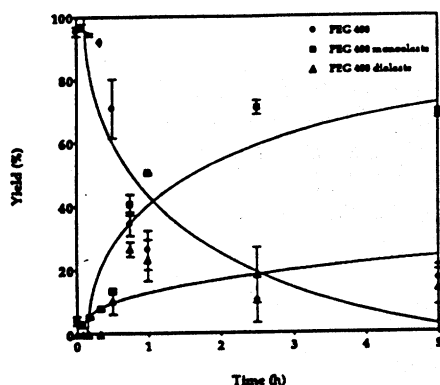


Fig. 3 Short time course of the production of PEG 400 ester at 42 °C, 50 mM PEG 400, 250 mM oleic acid.

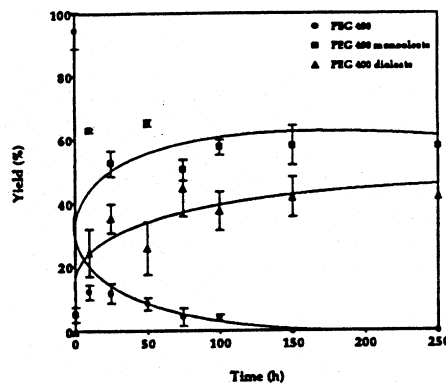


Fig. 4 Time course study of the production of PEG 400 esters at 50 °C in the presence of 50 mM PEG 400, 250 mM oleic acid.

Table 2. Effect of Lipozyme Recycling on Residual Activity^a.

Cycle #	Acylation Yield
	% ^b
1	100 ± 0.3
2	89 ± 2.5
3	85 ± 0.7
4	77 ± 7.8
5	80 ± 3.4

^a Recycling of Lipozyme was done as follows: the reaction conditions for each cycle were those described in Materials and Methods; each run was performed for 50 hrs at 42 °C. After each cycle the reaction solution was removed, and the Lipozyme powder was rinsed with excess methylene chloride, filtered over Whatman # 12 paper, and the solvent was evaporated under a stream of nitrogen before being placed in a new screw-capped tube containing fresh substrates.

^b All experiments were conducted twice in triplicate. The listed values are the averages and have been normalized with respect to the results obtained during cycle #1 (100%).

The ability of Lipozyme to retain its synthetic activity during recycling was examined. After five cycles high levels of esterification (80%) were still achieved (Table 2). Contrary to what had been observed with non-immobilized lipases in aqueous media using high concentrations of lower chain glycols, there was little enzyme denaturation (Okumura *et al.*, 1979).

No formation of the PEG esters could be observed at 42 °C in the absence of solvent or enzyme, or when hexane was replaced with aqueous buffer (Tris•HCl 0.1M, pH 6).

Conclusion

In this work we have acylated with success a longer chain glycol ($\text{HO}-(\text{C}_2\text{H}_4\text{O})_n\text{H}$, $n=9$) than previously reported, using a methodology that is both rapid and easy to handle. The complete esterification of PEG 400 using oleic acid as acyl donor at a relatively low temperature (42 °C) and Lipozyme as catalyst was achieved in hexane. By controlling the time of reaction, it was possible to direct the esterification reaction towards the preferential formation of PEG monooleate or PEG dioleate.

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